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ENHANCEMENT OF OLIGODENDROCYTE DIFFERENTIATION

(English)
(אנגלית:

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הגברת דיפרנציאציה לאוליגודנדרוציטים

ENHANCEMENT OF OLIGODENDROCYTE DIFFERENTIATION

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Ref: IL/880
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FIELD OF THE INVENTION

The present invention is generally in the field of neurological diseases and disorders. In particular it relates to neurodegenerative diseases in which the myelin cover of nerves is lost, as a result of degeneration of oligodendrocytes which are the cells producing the myelin in the central nervous system. More particularly, the present invention provides for the use of IL6R/IL6 chimera to promote the formation of oligodendrocytes from embryonic stem cells and as a medicament for treatment of neurodegenerative diseases or posttraumatic nerve damage.

BACKGROUND

Oligodendrocytes, which make the myelin sheaths in central nervous system (CNS), evolve from multipotential neural stem cells through a series of developmental stages (Rogister et al. 1999; Shihabuddin et al. 1999; Levine et al. 2001)for recent reviews). Recognized stages include early bipolar progenitors $A2B5^+$ cells, or O-2A (Raff 1989), late multipolar progenitors expressing the O4 sulfatide glycosides (Schachner et al. 1981), arborized immature oligodendrocytes O4 and GalC positive, and mature oligodendrocytes having the O1 sulfatide and synthesizing the myelin membrane with its structural components such as myelin basic protein (MBP).

Embryonic stem (ES) cell lines, derived from the inner cell mass of blastocyst-stage embryos, are a potential large scale source of oligodendrocytes and precursors derived from murine ES cell have been used for transplantion into myelin deficient CNS (Brustle et al. 1997; Brustle et al. 1999; McDonald et al. 1999). A number of culture conditions have been defined under which murine ES cells differentiated into floating embryoid bodies (EB) may be directed toward the neural lineages giving rise to various types of neurons, to astrocytes and to oligodendrocytes. One approach is

based on selection in serum-free defined medium in which neural precursor cells survive, proliferate under the influence of basic fibroblast growth factor (FGF-2) and differentiate upon growth factor removal and plating on adherent substrates (Okabe et al. 1996). Under these conditions, some O4 positive cells develop provided tri-iodothyronine (T3) is added, in line with T3 effect on optic nerve derived O-2A progenitors (Barres et al. 1994). A more efficient selection is obtained by sequential treatment of EB cells by FGF-2, then FGF-2 with epidermal growth factor (EGF), and FGF-2 with Platelet derived growth factor PDGF-AA, a factor promoting proliferation of glial precursor cells (Besnard et al. 1987; Bogler et al. 1990), thereby increasing the number of $A2B5^+$ cells which after growth factors withdrawal differentiate into both $O4^+$ oligodendrocytes and astrocytes expressing glial fibrillary acidic protein GFAP (Brustle et al. 1999).

Another approach uses differentiation agents such as retinoic acid to induce neural and glial lineages in EB cultures (Bain et al. 1995; Fraichard et al. 1995). As in newborn brain derived cultures, neural precursors can be further enriched by selecting non-adherent cells growing as floating spheres in defined medium, and expanding them as neurospheres and oligodendrocyte-enriched oligospheres that differentiate after EGF, FGF removal (Zhang et al. 1998; Liu et al. 2000). Human ES cell lines derived EBs also form neural tube like rosettes expandable as floating neurospheres that can be transplanted in vivo or plated on polycationic substrates to differentiate into neurons, astrocytes and oligodendrocytes, the latter developing particularly after treatment with PDGF-AA and T3 (Reubinoff et al. 2001; Zhang et al. 2001). Although cytokines such as LIF are known to maintain ES cells in an undifferentiated, multipotent state, it was recently found that LIF allows sparse murine ES cell cultures to develop into neurospheres (Tropepe et al. 2001).

Enriched murine ES cell derived oligospheres, yielding over 90% oligodendrocytes, have been obtained in a complex medium including combinations of hormones, such as T3 and progesterone, and cytokines such as Neurotrophin-3 (NT3) and ciliary neurotrophic factor CNTF (Liu et al. 2000). Both cytokines may contribute to an effect on oligodendrocyte precursors, as observed in optic nerve (Barres et al. 1994; Barres et al. 1996). However, the effects of CNTF on oligodendrocyte differentiation

are not clear as in some conditions it mainly induces $GFAP^+$ astrocytes from $A2B5^+$ progenitors (or earlier glioblasts) with little effect on $O4^+$ cells (Lillien et al. 1990; Gard et al. 1995; Johe et al. 1996; Bonni et al. 1997), whereas in other conditions it also increases survival and proportion of $GalC^+$, $O1^+$ and MBP^+ cells in the cultures (Kahn et al. 1994; Mayer et al. 1994; Marmur et al. 1998).

CNTF belongs to the interleukin-6 (IL-6) family of cytokines that signal via gp130 either as a heterodimeric receptor with LIF-R (for CNTF, LIF, OSM) or as a homodimer (for IL-6, IL-11) (Taga et al. 1997) for review). There is growing evidence on the importance of gp130 signaling for myelinating cells. In mice, postnatal gene deletion has indicated that gp130 is required to maintain Schwann cell function and myelination in peripheral nerves, in addition to its role in astrocytosis (Betz et al. 1998; Nakashima et al. 1999). With the help of a potent gp130 activating ligand, the IL6R/IL6 chimera in which IL-6 is fused to the extracellular portion of the IL-6 receptor (Chebath et al. 1997), we have previously observed induction of myelin gene expression in embryonic Schwann cells (Haggiag et al. 1999; Haggiag et al. 2001) and activation of myelin gene promoters (Slutsky et al. 2003). Activation of a transgenic MBP gene promoter in mice brain cultures was observed in response to CNTF (Stankoff et al. 2002) and in similar cortical cultures from newborn rat IL6R/IL6 chimera was more effective than CNTF to increase the development of highly arborized GalC+ oligodendrocytes (Valerio et al. 2002).

Promising results have been recently obtained in mice where injections of neural stem cells from the periventricular zone of adult mice brain and grown into neurospheres have induced clinical recovery and remyelination in an animal model of multiple sclerosis (Pluchino et al. 2003). Applying such technology to human patients suffering from multiple sclerosis or other demyelinating diseases, poses many difficulties because the neural stem cells would have to be isolated from cadavers or from aborted fetuses. Hence, the amount of cells that could be obtained would be limited, it would be difficult to ascertain that the brain cells do not transfer dangerous pathogens, and the transplants may cause problems of immuno-histocompatibility and may be rejected.

As indicated, blastocyst-derived ES cell lines, that are indefinitely expandable in laboratory tissue culture conditions, could provide a large-scale source of developing oligodendrocytes capable of myelinating neurons and thereby repairing lesions in the CNS (Cao et al. 2002; Gottlieb 2002).

Therefore, there is a need for a method to promote oligodendrocyte generation from ES cell lines.

SUMMARY OF THE INVENTION

The invention relates to a culture medium suitable for promoting differentiation of embryonic stem (ES) cells into oligodendrocytes comprising one or more gp 130 activators, and a solution suitable for culturing cells selected from ES, embryoid body (EB) and/or neurosphere (NS).

In addition, the invention provides a method of improving the potential of ES, EB and/or NS cell cultures for treatment of patients in order to repair damage caused by demyelinating diseases, comprising administrating to the cells one or more gp130 activators. Moreover, the invention provides an oligodendrocyte and/or oligodendrocyte precursors obtainable by this method and a method for treatment of demyelinating diseases comprising the administration of an effective amount of such oligodendrocytes.

In another aspect, the invention relates to a method for treatment of demyelinating diseases e.g. multiple sclerosis, stroke, spinal cord injury, and other trauma, demyelination of axon, comprising the administration of an effective amount of one or more gp 130 activator and ES, EB and /or NS cells in a patient in need.

The invention also provides a method for the preparation of differentiated oligodendrocytes for transplantation into patients in order to repair damage caused by demyelinating diseases comprising contacting, in vivo or ex-vivo, one or more gp 130 activator with ES, EB and/or NS cells.

The invention also provides the use of one or more gp 130 activators, in the manufacture of a medicament for generating oligodendrocytes from ES, EB and/or NS cells, to increase myelination of nerves in injured central neural system (CNS) usually caused by demyelination of nerves.

In one aspect the invention relates to the use of one or more gp 130 activators and ES, EB and/or NS cells in the manufacture of a medicament for the treatment of demyelinating diseases.

In another aspect, the invention discloses a pharmaceutical composition comprising one or more gp 130 activators and ES, EB and/or NS cells and more specifically for the treatment of a demyelinating disease.

In one embodiment, the invention relates to gp 130 activators such as LIF, CNTF, CT-1, OSM, IL-11, preferably IL-6, and more preferably IL6R/IL6 chimera, a mutein, functional derivative, active fraction, circularly permutated derivative or salt thereof. The gp 130 activator can be administered via an expression vector, a recombinant cell expressing the activator, preferably ES, EB and/or NS cells. The activator could be contacted with the cells ex-vivo or in-vivo.

In another embodiment of the invention, oligodendrocytes obtainable according to the invention can be implantated directly in the CNS or injected IV in a patient in need and allowed to migrate to the CNS.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows fluorescent microphotographies of representative fields from murine ES cells (R11 cell line) that were grown into neurospheres and then cultured either without (non-treated) or with IL6R/IL6 chimera (200 ng/ml) for 6 weeks, at which time the cells were fixed and stained with antibodies specific for oligodendrocytes. The upper panels show staining with monoclonal antibody O4, which specifically detects a sulfatide glycoside present in immature or pre-oligodendrocytes. The lower panels show staining

with anti-MBP antibodies against the myelin basic protein, which is a structural component of myelin formed only by mature oligodendrocytes. In the non-treated cultures, small $O4^+$ oligodendrocytes are observed whereas in the presence of IL6R/IL6 chimera there is a much more developed network of arborized oligodendrocytes, with long branches forming numerous neuronal contacts. In the IL6R/IL6 chimera -treated cultures, MBP is present in the branched network indicating the maturation of oligodendrocytes, contrasting with a weak staining of round and small cell bodies in the untreated cultures.

DETAILED DESCRIPTION OF THE INVENTION

The invention relates to the use of a gp 130 activator, such as IL6R/IL6 chimera, for generating oligodendrocytes from embryonic stem (ES), embryoid bodies (EB) and/or neurosphere (NS) cells in order to increase myelination of nerves in injured central nervous system (CNS).

The invention further relates to a method for improving the potential of embryo stem (ES) cell cultures for subsequent transplantation and treatment in patients with neurodegenerative diseases caused by loss of the myelin sheaths around the nerves.

The invention relates to a culture medium suitable for promoting differentiation ES, EB and/or NS cells into oligodendrocytes wherein the culture medium comprise one or more gp130 activators and a solution suitable for culturing ES, EB and/or NS cells.

Specifically, the present invention can be used to treat neurodegenerative disorders such as multiple sclerosis, stroke, spinal cord injury, and other trauma, demyelination of axons (Gledhill et al. 1973; Griffiths et al. 1983; Blight 1985; Bunge et al. 1993). The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

The use of cellular therapy is growing rapidly, and is gradually becoming an important therapeutic modality in treatment of various disorders for example, neuronal cell therapy for neurodegenerative diseases. Promising results have been recently obtained in mice where injections of neural stem cells from the periventricular zone of mice brain and grown into neurospheres have induced clinical recovery and remyelination in an animal model of multiple sclerosis (Pluchino et al. 2003). Applying such technology to human patients suffering from multiple sclerosis or other demyelinating diseases, poses many difficulties because the neural stem cells would have to be isolated from cadavers or from aborted fetuses. Hence, the amount of cells that could be obtained would be limited, it would be difficult to ascertain that the brain cells do not transfer dangerous pathogens, and the transplants may cause problems of immuno-histocompatibility and may be rejected.

The use of ES cells derived transplants has many advantages over the use of fetal or adult brain cells. The ES cells have high potential for expansion in vitro, can be ascertained to be pathogen-free and histocompatibility could be achieved by maintaining banks of ES cell lines (as done for bone marrow transplantation) or by cloning (i.e. producing ES cells from blastocysts produced by nuclear transfer from one of the patient's own cell)(Lanza et al. 1999). Experimental transplantation to animals has been successfully achieved with neurospheres derived from murine ES cells (Brustle et al. 1999; Liu et al. 2000) and from human ES cells (Reubinoff et al. 2001; Zhang et al. 2001). This approach could be greatly improved by having a method to promote the differentiation of ES cells toward specific cell lineages, such as myelinating oligodendrocytes.

In one embodiment of the invention, ES cells have been used to prepare neurospheres (by a procedure already used for human ES cells (Zhang et al. 2001)), and the

neurosphere cells were then subjected to treatment by a pure recombinant human IL6R/IL6 chimera molecule produced in CHO cells (200 ng/ml) in a serum-free chemically defined medium (as described in example 1). In the presence of IL6R/IL6 chimera, the neurospheres that had been plated on poly-D-lysine and fibronectin developed a dense network of oligodendrocyte progenitors identified by the O4 sulfatide glycoside on their surface. In the cultures performed according to the state-of-the-art, without IL6R/IL6 chimera, only few and small oligodendrocytes developed. Hence, IL6R/IL6 chimera showed a specific effect on promoting the differentiation of the neurosphere cells toward the oligodendrocyte lineage.

In another embodiment it was shown that IL6R/IL6 chimera can lead to differentiation into oligodendrocyte progenitors (Rip+) also when administrated into short term cultures of ES cell-derived dissociated neurosphere cells (Example 3). Since progenitor cells may be better suited for transplantation than fully differentiated cells, this result supports the advantages of using IL6R/IL6 chimera to improve the ex-vivo preparation of cells that upon injection can migrate into the CNS and effect myelination.

It was also found that IL6R/IL6 chimera can lead to the maturation of oligodendrocytes that express structural components of myelin as the myelin basic protein MBP, and form myelin membranes typical of mature myelinating cells. This activity of IL6R/IL6 chimera indicates that it may be itself injected together with transplanted ES, EB and/or NS cells to promote their maturation in vivo and increase the efficacy of the repair of CNS demyelinating lesions.

The present invention embraces the use one or more gp 130 activator, in the manufacture of a medicament for inducing generation of oligodendrocytes from ES, EB and/or NS cells. The gp 130 activator may be added ex-vivo to cultures of ES, EB and/or NS cells, which are thereafter transplanted to a patient. Alternatively the gp130 activator could be injected to a patient before together or after injecting the cells in order to stimulate the in vivo differentiation of ES, EB and/or NS transplants. A preferred in vivo gp130 activator is IL-6 and more preferably IL6R/IL6 chimera. IL6R/IL6 chimera may also be applied for stimulating the generation of oligodendrocytes early progenitors from ES, EB and/or NS cells. Additional gp130

asctivators according to the invention are selected from LIF, CNTF, CT-1, OSM IL-6 and IL-11.

ES cells according to the invention are any cells that are pluripotent, capable of producing progeny that are derivatives of all three germline layers, regardless of whether they were derived from embryonic tissue, fetal tissue, or other sources. In particular, embryonic stem cells (ES) from primates comprise various types of cells, e.g. human embryonic stem (hES) cells, described by Thomson et al.(Thomson et al. 1998); embryonic stem cells from other primates, such as Rhesus stem cells (Thomson et al. 1995), marmoset stem cells (Thomson et al. 1996) and human embryonic germ (hEG) cells (Shamblott et al. 1998).

Embryonic stem (ES) cell lines for the use according to the invention can be obtained from blastocyst-stage embryos (Brustle et al. 1997; Brustle et al. 1999; McDonald et al. 1999). Traditionally, ES cells are cultured on a layer of feeder cells, typically fibroblasts derived from embryonic or fetal tissue. To prepare a feeder cell layer, cells are irradiated or otherwise treated to inhibit proliferation but permit synthesis of factors that support ES cells. LIF or related cytokines are often added to maintain ES pluripotency.

Culture conditions inducing the progression of such ES cells through a series of transitions that may culminate in the generation of functional differentiated neuronal and glial cells comprise the following steps: 1- expansion of undifferentiated ES cells, 2- generation of embryoid bodies that include primitive endoderm and ectoderm layers, 3- culturing embryoid bodies to select for neurospheres in defined medium with one or more growth factors, typically bFGF, 4- expanding the neurospheres in suspension, 5- induction of differentiation of the expanded neurospheres by the withdrawal of growth factors and/or by growing in adherent conditions.

The cells according to the present invention comprise cells derived from anyone of the above five steps.

Embryoid bodies can be generated in suspension culture according to the method described by Martin et al., (1975)"Differentiation of clonal lines of teratocarcinoma cells: Formation of embryoid bodies in vitro," (Martin et al. 1975).

Briefly, to form embryoid bodies, the clusters of ES cells are disengaged from the tissue culture plates. Methods for disengaging cells from tissue culture plates are known and include the use of enzymes, such as trypsin or papain, dispase or commercially available preparations.

Generally, the ES cells disengage from the tissue culture plates in clusters (e. g., aggregates of 10 to 50 or more cells). The clusters of ES cells are then dissociated to obtain a population of cells which includes a majority of individual cells. Methods for dissociating clusters of cells are likewise known. One method for dissociating cells includes mechanically separating the cells, for example, by repeatedly aspirating a cell culture with a pipet. Preferably, the ES cells are in an exponential growth phase at the time of dissociation to avoid spontaneous differentiation that tends to occur in an overgrown culture.

The dissociated ES cells are then cultured in ES1 media as described below. However, in contrast to the ES cell proliferation stage (in which the cells are grown on a tissue culture dish surface), the embryoid bodies can be generated in suspension. For example, the cells may be cultured on non-adherent bacterial culture dishes. In this stage, the cells are incubated for about 4 days to about 7 days. Preferably, the medium is changed every 1 to 3 days.

A number of specific culture conditions for ES cells differentiation from embryoid bodies (EB) were described and are incorporated inhere by reference.

One approach is based on selection in serum-free defined medium in which neural precursor cells survive, proliferate under the influence of basic fibroblast growth factor (FGF-2) and differentiate upon growth factor removal and plating on adherent substrates (Okabe et al. 1996). Under these conditions, some O4 positive oligodendrocyte precursor cells develop when tri-iodothyronine (T3) is added, in line with T3 effect on optic nerve derived O-2A progenitors (Barres et al. 1994).

EB cultures can be obtained also by another approach, which uses differentiation agents such as retinoic acid to induce neural and glial lineages in EB cultures (Bain et al. 1995; Fraichard et al. 1995).

As in newborn brain derived cultures, neural precursors can be further enriched by selecting non-adherent cells growing as floating spheres in defined medium, and expanding them as neurospheres and oligodendrocyte-enriched oligospheres that differentiate after EGF, FGF removal (Reynolds et al. 1996; Zhang et al. 1998; Liu et al. 2000). Human ES cell lines derived EBs also form neural tube like rosettes expandable as floating neurospheres that can be transplanted in vivo or plated on polycationic substrates to differentiate into neurons, astrocytes and oligodendrocytes, the latter developing particularly after treatment with PDGF-AA and T3 (Reubinoff et al. 2001; Zhang et al. 2001).

Any commercial growth medium, or cell formulation, suitable for culturing embryonic stem cells (e.g. ES-Cult ^{tm} medium from StemCell Technologies) can be employed in the present invention. Non limiting examples for solutions suitable for culturing the cells of the invention comprise Dulbecco's DMEM (preferably high glucose about 4.5 mg/ml) (Gibco/BRL) with about 0.1 mM non-essential amino acids, 1-5 mM glutamine or about 0.5 g/liter, 0.5-5 mM sodium pyruvate or about 0.11 g/liter, 0.01-0.5 mM or about 0.01 g/liter β-mercaptoethanol, 2-20 % fetal calf serum (FCS) and optionally 100 U/ml penicillin, 0.1 mg/ml streptomycin, and about 40 mg/ml leukemia inhibitory factor LIF, or DMEM high glucose (preferably high glucose about 4.5 mg/ml), 5-20% newborn calf serum (heat-inactivated), 1-5 mM or about 0.5 g/liter glutamine, and optionally 50 U/ml penicillin, 50 μg/ml streptomycin, or DMEM/F12 (1:1), 2-20% FCS, 1-5 mM or about 0.5 g/liter or 0.5-2% glutamine, 0.01-0.5 mM β-mercaptoethanol, 0.5-5 μg/ml heparin and 1-10 ng/ml FGF-2 or serum-free defined medium comprising DMEM/F12 with 1-50 μg/ml insulin, 10-200 mg/ml transferrin, 10-100 μM putrescine, 5-100 nM or 0.01 mg/l sodium selenite, 0.5-10 μg/ml heparin, 2-200 nM progesterone and 0-100 ng/ml of FGF-2 or DMEM/F12 with 1-10 μg/ml insulin, 10-200 μg/ml transferrin, 1-50 μg/ml putrescine, 0-50 ng/ml

selenite and 1-20 ng/ml progesterone , and optionally 50 μg/ml ascorbic acid (Vitamin C) .

In one embodiment of the present invention the ES cell line R11 is cultured for 3 days on a feeder layer in ES medium (see example 1 below) containing LIF. Cells are subcultured with trypsin, seeded and cultured for 2 days in ES1 medium (see example 1 below) lacking LIF but containing FGF. To induce embryoid bodies cells are detached from the feeder layer with dispase and cultured in ES1 medium without FGF for 4 days. The clumps (embryoid bodies, EB) were picked and cultured in serum free, chemically-defined EB medium (see example 1 below) supplemented with FGF for 8-10 days. The neurospheres produced were picked and transferred to non-adherent bacterial culture dishes in which the spheres grew in suspension for 7 days in EB medium supplemented with FGF. Following this step the neurospheres (NS) were transferred to fibronecting coated plates to grow in adherent mode employing defined differentiation medium.

A similar procedure can be employed using human ES cells to obtain EB and/or NS cells as described (Reubinoff et al. 2001; Zhang et al. 2001).

According to the present invention, a gp130 activator is added to the cultures of ES, EB and/or NS cells to promote formation of oligodendrocyte progenitors, or the gp130 activator with ES, EB and/or NS cells are administered to a patient suffering from demyelinating disease to enhance oligodendrocyte differentiation of ES, EB and/or NS cells, either alone or together with other growth or differentiation agents such as retinoic acid, EGF, PDGF etc.

It will be appreciated that ES cells are also available commercially and can be used according to this aspect of the present invention. Human ES cells can be purchased, e.g. from the NIH human embryonic stem cells registry (<http://escr.nih.gov>).

The cells with gp130 activator according to the invention can be implanted directly in the CNS or can be injected, for example intravenously (IV), and allowed to migrate to the CNS.

A preferred gp130 activator in accordance with the present invention is IL-6, and more preferred IL6R/IL6 chimera, which is active even in cells that have only gp130 on their surface and lack other receptors of the IL-6 cytokine family.

An "IL6R/IL6 chimera" (also called "IL6R/IL6" or "IL-6 chimera"), as used herein, is a chimeric molecule comprising a soluble part of the interleukin-6 receptor fused to all or a biologically active fraction of interleukin-6. The moieties of the chimeric protein can be fused directly, or they can be linked by any suitable linker, such as a disulfide bridge or a polypeptide linker. The linker may be a short linker peptide, which can be as short as 1 to 3 amino acid residues in length or longer, for example, 13 or 18 amino acid residues in length. Said linker may be a tripeptide of the sequence E-F-M (Glu-Phe-Met), for example, or a 13-amino acid linker sequence comprising Glu-Phe-Gly-Ala-Gly-Leu-Val-Leu-Gly-Gly-Gln-Phe-Met introduced between the amino acid sequence of the soluble IL-6 receptor and the IL-6 sequence. Examples of IL6R/IL6 chimeric molecules are known in the art and have been described in detail e.g. in WO 99/02552 or WO 97/32891.

The IL6R/IL6 chimera may be produced in any adequate eukaryotic or prokaryotic cell type, such as yeast cells, insect cells, bacteria, and the like. It is preferably produced in mammalian cells, most preferably in genetically engineered CHO cells as described in WO 99/02552. Whilst the protein from human origin is preferred, it will be appreciated by the person skilled in the art that a similar fusion protein of any other origin may be used according to the invention, as long as it retains the biological activity described herein.

The delivery of IL6R/IL6 chimera to the brain may also be carried out using a vector comprising the coding sequence or an IL6R/IL6 chimera, a mutein, fused protein, active fraction or circularly permutated derivative thereof. The vector comprises all regulatory sequences needed for expression of the desired protein in the human body, preferably in the brain, more preferably in the striatum. Regulatory sequences for expression vectors are known by the person skilled in the art. The invention thus also relates to the use of a vector comprising the coding sequence of IL6R/IL6 chimera for manufacture of a medicament for the treatment of CNS injuries.

Any expression vector known in the art may be used according to the invention. However, a lentivirally-derived vector may be particularly useful for the delivery of IL6R/IL6 chimera directly into the striatum. Such lentiviral vectors are known in the art. They are specifically described e.g. in (Kordower et al. 1999; Deglon et al. 2000; Bensadoun et al. 2001).

Alternatively, the ES cells of the present invention can be contacted with gp 130 activator expressing and optionally presenting cells (i.e., insoluble-membrane bound gp 130 activator). This can be effected by co-culturing the stem cells of the present invention with cells, which express a secreted or membrane-bound gp 130 activator. For example, fibroblast feeder cells, which are oftentimes-co-cultured with stem cells to support proliferation thereof in a non-differentiated state can express an gp 130 activator of interest, thereby performing a dual role i.e., growth support and increase differentiation of ES into oligodendrocytes.

Alternatively, the ES cells of the present invention can be transformed with an expression construct such as that described below in order to express the gp 130 activator or the active portion thereof in the ES cells.

In such cases, the expression construct includes a cis-acting regulatory element active in mammalian cells (examples below). The nucleic acid construct of the present invention can further include an enhancer, which can be adjacent or distant to the promoter sequence and can function in up regulating the transcription therefrom.

As mentioned the preferred gp 130 activator is IL6R/IL6 chimera or a mutein, isoform, fused protein, functional derivative, active fraction, circularly permutated derivative or salt thereof and can be used for the manufacture of a medicament for the generation of oligodendrocytes from ES cells for the treatment of CNS injuries.

The terms "treating" as used herein should be understood as preventing, inhibiting, attenuating, ameliorating or reversing any or all symptoms or cause(s) of demyelinating neurodegenerative diseases.

As used herein the term "muteins" refers to analogs of an IL6R/IL6 chimera, in which one or more of the amino acid residues of the naturally occurring components of IL6R/IL6 chimera are replaced by different amino acid residues, or are deleted, or one

or more amino acid residues are added to the original sequence of an IL6R/IL6 chimera, without changing considerably the activity of the resulting products as compared with the original IL6R/IL6 chimera. These muteins are prepared by known synthesis and/or by site-directed mutagenesis techniques, or any other known technique suitable therefore.

Muteins in accordance with the present invention include proteins encoded by a nucleic acid, such as DNA or RNA, which hybridizes to DNA or RNA, which encodes an IL6R/IL6 chimera, in accordance with the present invention, under stringent conditions. The term "stringent conditions" refers to hybridization and subsequent washing conditions, which those of ordinary skill in the art conventionally refer to as "stringent". See Ausubel et al., Current Protocols in Molecular Biology, supra, Interscience, N.Y., §§6.3 and 6.4 (1987, 1992), and Sambrook et al. (Sambrook, J. C., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

Without limitation, examples of stringent conditions include washing conditions 12° °-20C below the calculated Tm of the hybrid under study in, e.g., 2 x SSC and 0.5% SDS for 5 minutes, 2 x SSC and 0.1% SDS for 15 minutes; 0.1 x SSC and 0.5% SDS at 37°C for 30-60 minutes and then, a 0.1 x SSC and 0.5% SDS at 68°C for 30-60 minutes. Those of ordinary skill in this art understand that stringency conditions also depend on the length of the DNA sequences, oligonucleotide probes (such as 10-40 bases) or mixed oligonucleotide probes. If mixed probes are used, it is preferable to use tetramethyl ammonium chloride (TMAC) instead of SSC. See Ausubel, supra.

Any such mutein preferably has a sequence of amino acids sufficiently duplicative of that of an IL6R/IL6 chimera, such as to have substantially similar, or even better, activity to IL6R/IL6 chimera.

One characteristic activity of IL6R/IL6 chimera is its capability of binding to gp130. An ELISA type assay for measuring the binding of IL6R/IL6 chimera to gp130 has been described in detail in example 7 on page 39 of WO 99/02552, which is fully incorporated by reference herein. As long as the mutein has substantial binding activity to gp130, it can be considered to have substantially similar activity to

IL6R/IL6 chimera. Thus, it can be determined whether any given mutein has at least substantially the same activity as IL6R/IL6 chimera by means of routine experimentation comprising subjecting such a mutein, e.g., to a simple sandwich binding assay to determine whether or not it binds to an immobilized gp130, as described in example 7 of WO 99/02552.

In a preferred embodiment, any such mutein has at least 40% identity or homology with the amino acid sequence of IL6R/IL6 chimera comprised in WO 99/02552. More preferably, it has at least 50%, at least 60%, at least 70%, at least 80% or, most preferably, at least 90% identity or homology thereto.

Identity reflects a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, determined by comparing the sequences. In general, identity refers to an exact nucleotide to nucleotide or amino cid to amino acid correspondence of the two polynucleotides or two polypeptide sequences, respectively, over the length of the sequences being compared.

For sequences where there is not an exact correspondence, a "percent identity" may be determined. In general, the two sequences to be compared are aligned to give a maximum correlation between the sequences. This may include inserting "gaps" in either one or both sequences, to enhance the degree of alignment. A percent identity may be determined over the whole length of each of the sequences being compared (so-called global alignment), that is particularly suitable for sequences of the same or very similar length, or over shorter, defined lengths (so-called local alignment), that is more suitable for sequences of unequal length.

Methods for comparing the identity and homology of two or more sequences are well known in the art. Thus for instance, programs available in the Wisconsin Sequence Analysis Package, version 9.1 (Devereux J et al 1984, Nucleic Acids Res. 1984 Jan 11;12(1 Pt 1):387-95.), for example the programs BESTFIT and GAP, may be used to determine the % identity between two polynucleotides and the % identity and the % homology between two polypeptide sequences. BESTFIT uses the "local homology" algorithm of Smith and Waterman (J Theor Biol. 1981 Jul 21;91(2):379-80 and J Mol Biol. 1981 Mar 25;147(1):195-7. 1981) and finds the best single region of similarity

between two sequences. Other programs for determining identity and/or similarity between sequences are also known in the art, for instance the BLAST family of programs (Altschul S F et al, 1990 J Mol Biol. 1990 Oct 5;215(3):403-10, Proc Natl Acad Sci U S A. 1990 Jul;87(14):5509-13, Altschul S F et al, Nucleic Acids Res. 1997 Sep 1;25(17):3389-402, accessible through the home page of the NCBI at www.ncbi.nlm.nih.gov) and FASTA (Pearson W R, Methods Enzymol. 1990;183:63-98. Pearson J Mol Biol. 1998 Feb 13;276(1):71-84).

Muteins of IL6R/IL6 chimera, which can be used in accordance with the present invention, or nucleic acid coding therefore, include a finite set of substantially corresponding sequences as substitution peptides or polynucleotides which can be routinely obtained by one of ordinary skill in the art, without undue experimentation, based on the teachings and guidance presented herein.

Preferred changes for muteins in accordance with the present invention are what are known as "conservative" substitutions. Conservative amino acid substitutions of IL6R/IL6 chimera may include synonymous amino acids within a group which have sufficiently similar physicochemical properties that substitution between members of the group will preserve the biological function of the molecule (Grantham Science. 1974 Sep 6;185(4154):862-4). It is clear that insertions and deletions of amino acids may also be made in the above-defined sequences without altering their function, particularly if the insertions or deletions only involve a few amino acids, e.g., under thirty, and preferably under ten, and do not remove or displace amino acids which are critical to a functional conformation, e.g., cysteine residues. Proteins and muteins produced by such deletions and/or insertions come within the purview of the present invention.

Preferably, the synonymous amino acid groups are those defined in Table 1. More preferably, the synonymous amino acid groups are those defined in Table 2; and most preferably the synonymous amino acid groups are those defined in Table 3.

TABLE 1

Preferred Groups of Synonymous Amino Acids

Amino Acid	Synonymous Group
Ser	Ser, Thr, Gly, Asn
Arg	Arg, Gln, Lys, Glu, His
Leu	Ile, Phe, Tyr, Met, Val, Leu
Pro	Gly, Ala, Thr, Pro
Thr	Pro, Ser, Ala, Gly, His, Gln, Thr
Ala	Gly, Thr, Pro, Ala
Val	Met, Tyr, Phe, Ile, Leu, Val
Gly	Ala, Thr, Pro, Ser, Gly
Ile	Met, Tyr, Phe, Val, Leu, Ile
Phe	Trp, Met, Tyr, Ile, Val, Leu, Phe
Tyr	Trp, Met, Phe, Ile, Val, Leu, Tyr
Cys	Ser, Thr, Cys
His	Glu, Lys, Gln, Thr, Arg, His
Gln	Glu, Lys, Asn, His, Thr, Arg, Gln
Asn	Gln, Asp, Ser, Asn
Lys	Glu, Gln, His, Arg, Lys
Asp	Glu, Asn, Asp
Glu	Asp, Lys, Asn, Gln, His, Arg, Glu
Met	Phe, Ile, Val, Leu, Met
Trp	Trp

TABLE 2

More Preferred Groups of Synonymous Amino Acids

Amino Acid	Synonymous Group
Ser	Ser
Arg	His, Lys, Arg
Leu	Leu, Ile, Phe, Met
Pro	Ala, Pro
Thr	Thr
Ala	Pro, Ala
Val	Val, Met, Ile
Gly	Gly
Ile	Ile, Met, Phe, Val, Leu
Phe	Met, Tyr, Ile, Leu, Phe
Tyr	Phe, Tyr
Cys	Cys, Ser
His	His, Gln, Arg
Gln	Glu, Gln, His
Asn	Asp, Asn
Lys	Lys, Arg
Asp	Asp, Asn
Glu	Glu, Gln
Met	Met, Phe, Ile, Val, Leu
Trp	Trp

TABLE 3
Most Preferred Groups of Synonymous Amino Acids

Amino Acid	Synonymous Group
Ser	Ser
Arg	Arg
Leu	Leu, Ile, Met
Pro	Pro
Thr	Thr
Ala	Ala
Val	Val
Gly	Gly
Ile	Ile, Met, Leu
Phe	Phe
Tyr	Tyr
Cys	Cys, Ser
His	His
Gln	Gln
Asn	Asn
Lys	Lys
Asp	Asp
Glu	Glu
Met	Met, Ile, Leu
Trp	Met

Examples of production of amino acid substitutions in proteins which can be used for obtaining muteins of IL6R/IL6 chimera polypeptides, for use in the present invention include any known method steps, such as presented in US patents 4,959,314, 4,588,585 and 4,737,462, to Mark et al; 5,116,943 to Koths et al., 4,965,195 to Namen et al; 4,879,111 to Chong et al; and 5,017,691 to Lee et al; and lysine substituted proteins presented in US patent No. 4,904,584 (Shaw et al).

"Functional derivatives" as used herein cover derivatives of IL6R/IL6 chimera, and their muteins, which may be prepared from the functional groups which occur as side chains on the residues or are additions to the N- or C-terminal groups, by means known in the art, and are included in the invention as long as they remain pharmaceutically acceptable, i.e. they do not destroy the activity of the protein which is substantially similar to the activity of IL6R/IL6 chimera, and do not confer toxic properties on compositions containing it.

These derivatives may, for example, include polyethylene glycol side-chains, which may mask antigenic sites and extend the residence of an IL6R/IL6 chimera in body fluids. Other derivatives include aliphatic esters of the carboxyl groups, amides of the carboxyl groups by reaction with ammonia or with primary or secondary amines, N-acyl derivatives of free amino groups of the amino acid residues formed with acyl moieties (e.g. alkanoyl or carboxylic aroyl groups) or O-acyl derivatives of free hydroxyl groups (for example that of seryl or threonyl residues) formed with acyl moieties.

An "active fraction" according to the present invention may e.g. be a fragment of IL6R/IL6 chimera. The term fragment refers to any subset of the molecule, that is, a shorter peptide that retains the desired biological activity. Fragments may readily be prepared by removing amino acids from either end of the IL6R/IL6 chimera molecule and testing the resultant fragment for its properties to bind to gp130. Proteases for removing one amino acid at a time from either the N-terminal or the C- terminal of a polypeptide are known, and so determining fragments, which retain the desired biological activity, involves only routine experimentation.

As active fractions of an IL6R/IL6 chimera, muteins and fused proteins thereof, the present invention further covers any fragment or precursors of the polypeptide chain of the protein molecule alone or together with associated molecules or residues linked thereto, e.g., sugar or phosphate residues, or aggregates of the protein molecule or the sugar residues by themselves, provided said fraction has substantially similar activity to gp130.

The term "salts" herein refers to both salts of carboxyl groups and to acid addition salts of amino groups of the IL6R/IL6 chimera molecule or analogs thereof. Salts of a carboxyl group may be formed by means known in the art and include inorganic salts, for example, sodium, calcium, ammonium, ferric or zinc salts, and the like, and salts with organic bases as those formed, for example, with amines, such as triethanolamine, arginine or lysine, piperidine, procaine and the like. Acid addition salts include, for example, salts with mineral acids, such as, for example, hydrochloric acid or sulfuric acid, and salts with organic acids, such as, for example, acetic acid or

oxalic acid. Of course, any such salts must retain the biological activity of IL6R/IL6 chimera, i.e., the ability to bind to gp130.

The term "circularly permuted" as used herein refers to a linear molecule in which the termini have been joined together, either directly or through a linker, to produce a circular molecule, and then the circular molecule is opened at another location to produce a new linear molecule with termini different from the termini in the original molecule. Circular permutations include those molecules whose structure is equivalent to a molecule that has been circularized and then opened. Thus, a circularly permuted molecule may be synthesized de novo as a linear molecule and never go through a circularization and opening step. The particular circular permutation of a molecule is designated by brackets containing the amino acid residues between which the peptide bond is eliminated. Circularly permuted molecules, which may include DNA, RNA and protein, are single-chain molecules, which have their normal termini fused, often with a linker, and contain new termini at another position. See Goldenberg, et al. J. Mol. Biol., 165: 407-413 (1983) and Pan et al. Gene 125: 111-114 (1993), both incorporated by reference herein. Circular permutation is functionally equivalent to taking a straight-chain molecule, fusing the ends to form a circular molecule, and then cutting the circular molecule at a different location to form a new straight chain molecule with different termini. Circular permutation thus has the effect of essentially preserving the sequence and identity of the amino acids of a protein while generating new termini at different locations.

In a preferred embodiment of the invention, the IL6R/IL6 chimera is glycosylated at one or more sites.

A glycosylated form of an IL6R/IL6 chimera has been described in WO 99/02552 (PCT/IL98/00321), which is the chimeric molecule highly preferred according to the invention. The IL6R/IL6 chimera described therein is a recombinant glycoprotein, which was obtained fusing the entire coding sequence of the naturally occurring soluble IL-6 receptor δ-Val (Novick et al. 1990) to the entire coding sequence of mature naturally occurring IL-6, both from human origin.

The IL6R/IL6 chimera may be produced in any adequate eucaryotic or procaryotic cell type, like yeast cells, insect cells, bacteria, and the like. It is preferably produced in mammalian cells, most preferably in genetically engineered CHO cells as described in WO 99/02552. Whilst the protein from human origin is preferred, it will be appreciated by the person skilled in the art that a similar fusion protein of any other origin may be used according to the invention, as long as it retains the biological activity described herein.

Advantageously, the chimeric molecule can then be produced in bacterial cells, which are not capable of synthesizing glycosyl residues, but usually have a high yield of produced recombinant protein.

Functional derivatives of IL6R/IL6 chimera may be conjugated to polymers in order to improve the properties of the protein, such as the stability, half-life, bioavailability, tolerance by the human body, or immunogenicity.

Therefore, a preferred embodiment of the invention relates to a functional derivative of the IL6R/IL6 chimera comprising at least one moiety attached to one or more functional groups, which occur as one or more side chains on the amino acid residues.

A highly preferred embodiment relates to an IL6R/IL6 chimera linked to Polyethlyenglycol (PEG). PEGylation may be carried out by known methods, such as the ones described in WO 92/13095, for example.

The IL6R/IL6 chimera may be delivered to the brain in any adequate formulation. It may also be delivered in form of cells expressing and/or secreting an IL6R/IL6 chimera, a mutein, fused protein, active fraction or circularly permutated derivative thereof.

The invention therefore further relates to the use of ES cells and IL6R/IL6 chimera, a mutein, fused protein, active fraction or circularly permutated derivative thereof, for manufacture of a medicament for the treatment of CNS injuries. The cells may be administered in any suitable form. However, a polymer-encapsulated cell is a highly preferred mode of delivery of the cells. The encapsulation procedure is described in detail e.g. by Emerich et al (J Comp Neurol. 1994 Nov 1;349(1):148-64 and Exp

Neurol. 1994 Nov;130(1):141-50.) or US 5,853,385. Suitable cell lines and stable expression systems are well known in the art.

The delivery of IL6R/IL6 chimera to the brain may also be carried out using a vector comprising the coding sequence or an IL6R/IL6 chimera, a mutein, fused protein, active fraction or circularly permutated derivative thereof. The vector comprises all regulatory sequences needed for expression of the desired protein in the human body, preferably in the brain, more preferably in the striatum. Regulatory sequences for expression vectors are known by the person skilled in the art. The invention thus also relates to the use of a vector comprising the coding sequence of IL6R/IL6 chimera for manufacture of a medicament for the treatment of demyelinating diseases.

Any expression vector known in the art may be used according to the invention. However, a lentivirally-derived vector may be particularly useful for the delivery of IL6R/IL6 chimera directly into the striatum. Such lentiviral vectors are known in the art. They are specifically described e.g. in Kordower et al. (1999) or Déglon et al. (2000).

It is a further object of the present invention to provide a pharmaceutical composition comprising IL6R/IL6 chimera, a mutein, fused protein, functional derivative, active fraction, circularly permutated derivative or salt thereof, optionally together with one or more pharmaceutically acceptable carriers, diluents or excipients, for the treatment of CNS injury. The IL6R/IL6 chimera used may be either from eukaryotic origin (glycosylated) or from bacterial origin (non-glycosylated).

The invention further relates to a pharmaceutical composition comprising IL6R/IL6 chimera, to a pharmaceutical composition comprising an expression vector, in particular a lentiviral gene therapy vector expressing IL6R/IL6 chimera and to pharmaceutical composition comprising in addition to the IL6R/IL6 chimera (protein or vector) ES cells optionally together with one or more pharmaceutically acceptable carriers, diluents or excipients, for the treatment of CNS injury.

The pharmaceutical composition, according to the invention, may comprise a mixture of gp 130 activators.

The definition of "pharmaceutically acceptable" is meant to encompass any carrier, which does not interfere with effectiveness of the biological activity of the active ingredient and that is not toxic to the host to which it is administered. For example, for parenteral administration, IL6R/IL6 chimera may be formulated in a unit dosage form for injection in vehicles such as saline, dextrose solution, serum albumin and Ringer's solution.

The IL6R/IL6 chimera can be administered to a patient in need in a variety of ways. The routes of administration include intracranial, intradermal, transdermal (e.g. in slow release formulations), intramuscular, intraperitoneal, intravenous, subcutaneous, oral, epidural, topical, and intranasal routes. Any other therapeutically efficacious route of administration can be used, for example absorption through epithelial or endothelial tissues or by gene therapy wherein a DNA molecule encoding the IL6R/IL6 chimera is administered to the patient (e.g. via a vector), which causes the IL6R/IL6 chimera to be expressed and secreted in vivo. In addition the IL6R/IL6 chimera can be administered together with other components of biologically active agents such as pharmaceutically acceptable surfactants, excipients, carriers, diluents and vehicles.

For parenteral (e.g. intravenous, subcutaneous, intramuscular) administration, IL6R/IL6 chimera can be formulated as a solution, suspension, emulsion or lyophilized powder in association with a pharmaceutically acceptable parenteral vehicle (e.g. water, saline, dextrose solution) and additives that maintain isotonicity (e.g. mannitol) or chemical stability (e.g. preservatives and buffers). The formulation is sterilized by commonly used techniques.

It is a further object of the present invention to provide for a method for treating demyelinating diseases, comprising administering to a patient in need thereof an effective amount of IL6R/IL6 chimera, a mutein, fused protein, functional derivative, active fraction, circularly permutated derivative or salt thereof optionally together with a pharmaceutically acceptable carrier and effective amount of ES, EB, NS and/or derived cells.

An "effective amount" refers to an amount of the active ingredients that is sufficient to affect the course and the severity of the diseases described above, leading to the reduction or remission of such pathology. The effective amount will depend on the route of administration and the condition of the patient.

The dosage administered, as single or multiple doses, to an individual will vary depending upon a variety of factor, including IL6R/IL6 chimera pharmacokinetic properties, the route of administration, patient conditions and characteristics (sex, age, body weight, health, size), extent of symptoms, concurrent treatments, frequency of treatment and the effect desired. Adjustment and manipulation of established dosage ranges are well within the ability of those skilled.

A method for treating demyelinating diseases, comprise administering to a patient in need thereof an effective amount of IL6R/IL6 chimera, a mutein, fused protein, active fraction or circularly permutated derivative thereof, or comprising administering to a patient in need thereof an expression vector comprising the coding sequence of IL6R/IL6 chimera, a mutein, fused protein, active fraction or circularly permutated derivative thereof, and ES cells.

It is a further object of the present invention to provide for a method for the preparation of differentiated oligodendrocytes for transplantation into patients in order to repair damage caused by demyelinating diseases. The IL6R/IL6 chimera will in this case be used ex-vivo to stimulate the development of oligodendrocytes from ES cells. Such stimulation can greatly improve the yield of oligodendrocyte cells from in vitro cultures, facilitating the use of these tissues for subsequent transplantation.

The present invention will now be described in more detail in the following non-limiting examples and the accompanying drawings.

EXAMPLES

Example 1: IL6R/IL6 chimera enhances the differentiation of oligodendrocyte progenitors expressing the O4 sulfatide.

The murine ES cell line Rosa 11 (R11) (Li et al. 2001) was seeded at $0.7x10^6$ cells/ 6cm dish on top of feeder layer of γ-irradiated embryo fibroblasts (see below) in 5 ml of ES medium comprised of Dulbecco's DMEM high glucose (4.5 mg/ml) (Gibco/BRL) with 0.1 mM non-essential amino acids, 2 mM glutamine, 1mM sodium pyruvate, 0.1 mM β-mercaptoethanol, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 15% fetal calf serum (FCS) and 40 mg/ml leukemia inhibitory factor LIF, and cultured 3 days. All cultures were at 37°C in 6.5% CO_2, with daily replacement of medium. The feeder cells were obtained by trypsinisation of 15.5 day old mouse embryos, cultured at $5x10^6$ cells/10 cm plate in 10 ml of DMEM high glucose (4.5 mg/ml), 10% newborn calf serum (heat-inactivated at 56°C , 0.5 hour), 2 mM glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, trypsinized and suspended in 5 ml of same medium for γ-irradiation from a cobalt source at 3000 rad for 10.5 minutes, and then plated at $0.3x10^6$ cells/6 cm dish.

After 3 days, the R11 ES cells were subcultured with 0.05% trypsin and seeded as above but in ES1 medium comprising DMEM/F12 (1:1), 15% FCS, 1% glutamine, 0.1 mM β-mercaptoethanol, 2 μg/ml heparin and 4 ng/ml FGF-2. After 2 days, the ES cells treated with 0.2 mg/ml dispase (Gibco/BRL) at 37°C for 15 minutes, and the detached cell clusters were washed and cultured in 3 tissue culture (Nunclon) 9cm-plates with 10 ml of medium ES1 but without FGF, for 4 days during which embryoid bodies (EB) developed. These were then treated with 0.1 mg/ml dispase (10 min 37°C) and the dislodged EBs were picked and transferred to new plates (about 50 /25 cm^2) in serum-free defined EB medium comprising DMEM/F12 with 25 μg/ml insulin, 100 μg/ml transferrin, 60 μM putrescine, 30 nM sodium selenite, 2 μg/ml heparin, 20 nM progesterone and 20 ng/ml of FGF-2, and cultured 8-10 days with daily medium change. The cultures were again partially dissociated with 0.1 mg/ml dispase (10 min 37°C) and ~200 spherical clumps (identified as neurospheres, NS, by the outgrowth of axons) were picked and transferred into non-adherent bacterial

culture Petri dishes (9 cm with 10 ml of the same EB medium) in which the spheres grew in suspension for 7 days.

The neurospheres were deposited on individual glass cover slips that had been coated with a solution of 20 μg/ml poly-D-lysine (Sigma, St Louis, MO)), applied overnight at 37°C in 5% CO_2, washed in water and dried before adding 250 μg/ml fibronectin for 2-12 hours at 4°C. About 4 neurospheres were placed on each coated coverslip, which were put at the bottom of each well of 12-well plates in 1 ml of defined differentiation medium containing DMEM/F12 with 5 μg/ml insulin, 100 μg/ml transferrin, 16.1 μg/ml putrescine, 5.2 ng/ml selenite and 6.3 ng/ml progesterone (supplied as 1% N2 supplement, Gibco/BRL). Half of the wells were supplemented with pure IL6R/IL6 chimera, 200 ng/ml (volume of stock added to each well) (produced in CHO cells as described in (Chebath et al. 1997)). The medium and all its ingredients were replaced every 3 days and after 3 weeks 50 μg/ml ascorbic acid (Vitamin C) was added to the medium and the culture continued. At 6 weeks after plating the neurospheres, the cultures were fixed in 4% paraformaldehyde and kept in PBS at 4°C. Before immunostaining, the plates were blocked with 5% FCS in PBS for 30 min at 22°C. Staining for immature oligodendrocytes was done with anti-sulfatide O4 monoclonal antibodies (Chemicon, Temecula, CA; used at 1:75 for 60 min at 37°C) and FITC-conjugated anti-IGM polyclonal antibodies (at 1:50 for 60 min at 37°C). Monoclonal antibody TuJ-1 anti-tubulin beta III (Covance Research Products, Berkeley, CA, diluted 1:400) was used to stain the axonal network. Astrocytes were stained with monoclonal anti glial fibrillary acid protein (GFAP) conjugated with the fluorescent tag Cy3 (Sigma, St Louis, MO), 1:400). After washing with PBS 3 times for 5 min, the secondary antibody, FITC- or Cy3-conjugated goat anti-mouse IgG or IgM (Jackson ImmunoResearch Lab, Inc, 1:400 in PBS) was added and left for 1h at room temperature, before washing with PBS 3 times for 5 minutes, and mounting in Mowiol (Calbiochem, LaJolla,CA). Samples were examined with a microscope Olympus IX-70 FLA under UV-light fluorescence. Photographs were made with a microscope mounted DVC digital camera and were processed in Photoshop.

Using the above procedure, the effect of IL6R/IL6 chimera on neurospheres cell differentiation was studied. In brief, murine R11 ES cells, which had been subcultured on a feeder layer of irradiated embryo fibroblasts in medium with serum and FGF-2,

were removed from the feeder layer, cultured 4 days in tissue culture dishes without FGF-2 and the resulting EBs were dissociated and re-plated in serum-free defined medium with 20 ng/ml FGF-2 for 8 days. As described (Zhang et al. 2001), rosette-type clumps that may represent neural tube-like structures appeared and were dislodged by partial digestion with dispase to be transferred as suspension cultures in non-adherent plates in the same medium. Within a week, the clumps grew to form neurospheres that were picked and adhered onto glass cover slips coated with poly-D-lysine and fibronectin, a substrate that favors glial cell development (Reubinoff et al. 2001). Numerous neuronal processes grew out of the spheres, forming axonal bundles that were visualized by immunostaining for βIII-tubulin. At 3 weeks, the axonal network was comparable in the outgrowth of neurospheres plated either with or without IL6R/IL6 chimera (not shown). Astrocytes, stained for GFAP, were present among the neuronal bundles in the control cultures but were consistently more abundant and more elongated in the IL6R/IL6 chimera treated cultures (not shown).

The more striking difference was observed when pro-oligodendrocytes or immature oligodendrocytes were visualized by staining with anti-sulfatide O4 antibodies. At 6 weeks, the control cultures showed spreading of a number of multipolar $O4^+$ cells among the underlying layer of cells outgrowing from the neurosphere (Figure, upper left panel). In contrast, the cultures with IL6R/IL6 chimera contained a dense network of $O4^+$ cells with considerably more arborization, which formed the majority of the cells in certain areas of the outgrowth and surrounded thickened nerve fibers (Figure upper right panel). When observed individually, O4 stained oligodendrocytes in the IL6R/IL6 chimera treated cultures could be seen to have grown to a much larger size than in the control cultures.

Example 2: IL6R/IL6 chimera enhances the maturation of oligodendrocytes to the myelinating stage

The same procedure as in Example 1 was used, except that the cells growing out from neurospheres were stained with monoclonal antibodies against the myelin basic protein MBP (MAB 386, Chemicon). The accumulation of MBP, a structural myelin protein was clearly observed in the oligodendrocyte network of IL6R/IL6 chimera -treated

cultures, but not in the corresponding control cultures (Figure lower panels). The gp130 activator, therefore, not only increased the number and density of the ES-cell derived oligodendrocytes but also their maturation toward the myelinating phenotype. This maturation was further denoted in the IL6R/IL6 chimera -treated cultures by the development of flattened myelin-like membrane sheaths that characterize myelinating oligodendrocytes. This was not seen in the untreated cultures.

The maturation of oligodendrocytes is also characterized by the appearance of the O1 sulfatide glycoside, which replaces the O4-sulfatide seen in immature cells (Schachner et al. 1981). Large $O1^+$ oligodendrocytes with extensive arborization were seen in the IL6R/IL6 chimera -treated cultures, whereas only few and small cells could be found in the corresponding control cultures (not shown).

Example 3: IL6R/IL6 chimera increases the number of oligodendrocyte progenitors in short term dissociated cell cultures

Prolonged maintenance of ES cells on the feeder layer gives rise to cell clumps which are enriched for neural progenitors and can be mechanically isolated (Reubinoff et al. 2001). The clumps form floating neurospheres upon culture in serum-free defined EB medium (conditions described in Example 1). Such spheres, prepared from R11 ES cells, were then digested with 5 mg/ml of collagenase/ dispase mixture (Sigma, #C3180, St Louis, MO) and the dissociated cells were seeded on poly-D-lysine and fibronectin cover slips at a density of 1.5 $x10^4$ cells/well in 12 well plates. Culture was in defined differentiation medium as in Example 1, FGF-2 and 5 μg/ml laminin being added for the first 4 days and then removed. At this time, half of the wells were supplemented with IL6R/IL6 chimera, 200 ng/ml, and the cultures continued for 18 days. The fixed cells were stained with the Rip monoclonal antibody (from the hybridoma bank, University of Iowa) which labels oligodendrocytes from early stages of development to mature cells but does not stain astrocytes (Friedman et al. 1989). In the cultures treated with IL6R/IL6 chimera, colonies of Rip^+ oligodendrocytes were of much larger size and density than in the untreated cultures (not shown). In these relatively short-term cultures of dissociated cells, the oligodendrocytes had short multipolar processes indicating that they are still at the progenitor stage. Hence, IL6R/IL6 chimera acts early on the

differentiation of this cell lineage, and would allow to obtain large amounts of ES cell derived oligodendrocyte progenitors suitable for transplantation.

Example 4: IL6R/IL6 chimera enhances the differentiation of human oligodendrocyte progenitors

The human EB and neurospheres are prepared as described by Zhang et al (2001) Briefly, human ES cell lines derived from H1 and H9.2 (as described by Amit et al. 2000) are propagated on a feeder layer of irradiated mouse embryonic fibroblasts (as described by (Thomson et al. 1998). To initiate differentiation, ES colonies are detached and grown as embryoid bodies for four days. The EB are then cultured in a tissue culture treated flask in a chemical defined medium, as described by (Zhang et al. 1999; Zhang et al. 2000; Zhang et al. 2001) . After five to seven days in culture with FGF the EB cells generate flattened cells and also an increasing number of small, elongated cells. By seven days the small elongated cells generate rosette formations (or neurospheres). Treatment of dispase leads to preferential detachment of the neurospheres.

The neurospheres are picked and adhered onto glass cover slips coated with poly-D-lysine and fibronectin, a substrate that favors glial cell development (Reubinoff et al. 2001) and supplemented with pure IL6R/IL6 chimera, 200 ng/ml as described in Example 1. Numerous neuronal processes grew out of the spheres, forming axonal bundles that is visualized by immunostaining for βIII-tubulin.

Pro-oligodendrocytes or immature oligodendrocytes are visualized by staining with anti-sulfatide O4 antibodies. After 2-6 weeks, the control cultures (without IL6R/IL6 chimera) show spreading of a number of small multipolar $O4^+$ cells among the underlying layer of cells outgrowing from the neurosphere. In contrast, the cultures with IL6R/IL6 chimera contain a dense network of $O4^+$ cells with considerably more arborization. Staining with antibodies for MBP demonstrates that the IL6R/IL6 chimera promotes the myelinating activity of the cells.

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Claims

1. A culture medium suitable for promoting differentiation of embryonic stem (ES) cells into oligodendrocytes comprising one or more gp 130 activators and a solution suitable for culturing cells selected from ES, embryoid body (EB) and/or neurosphere (NS).

2. A culture medium according to claims 1, wherein the solution is suitable for culturing ES.

3. A culture medium according to claims 1, wherein the solution is suitable for culturing EB.

4. A culture medium according to claims 1, wherein the solution is suitable for culturing NS.

5. A culture medium according to claim 1, wherein the gp 130 activator is selected from LIF, CNTF, CT-1, OSM IL-6, IL6R/IL6 chimera and IL-11.

6. A culture medium according to claim 5, wherein the gp 130 activator is IL6R/IL6 chimera, a mutein, functional derivative, active fraction, circularly permutated derivative or salt thereof.

7. A method of improving the potential of ES, EB and/or NS cell cultures for treatment of patients in order to repair damage caused by demyelinating diseases comprising administrating to the cells one or more gp130 activators.

8. A method according to claim 7, wherein the cells comprise ES cells.

9. A method according to claim 7, wherein the cells comprise EB cells.

10. A method according to claim 7, wherein the cells comprise NS.

11. The method according to claim 7, wherein the gp 130 activator is selected from LIF, CNTF, CT-1, OSM IL-6, IL6R/IL6 chimera and IL-11.

12. The method according to claim 11, wherein the gp 130 activator comprises IL6R/IL6 chimera, a mutein, functional derivative, active fraction, circularly permutated derivative or salt thereof.

13. The method according to claim 7, wherein the demyelinating disease is selected from multiple sclerosis, stroke, spinal cord injury, and other trauma, demyelination of axon.

14. An oligodendrocyte and/or oligodendrocyte precursors obtainable by a method according to anyone of claims 7 to 13.

15. A method for treatment of demyelinating diseases comprising the administration of an effective amount of the oligodendrocytes according to claim 14.

16. The method according to claim 15, wherein the cells are implantated directly in the CNS.

17. The method according to claim 15, wherein the cells are IV injected and allowed to migrate to the CNS.

18. A method for treatment of demyelinating diseases comprising the administration of an effective amount of one or more gp 130 activator and ES, EB and /or NS cells in a patient in need.

19. The method according to claim 18, comprising the administration of ES cells.

20. The method according to claim 18, comprising the administration of EB cells.

21. The method according to claim 18, comprising the administration of NS cells.

22. The method according to claim 18, wherein the gp 130 activator is selected from LIF, CNTF, CT-1, OSM IL-6, IL6R/IL6 chimera and IL-11.

23. The method according to claim 22, wherein the gp 130 activator is administrated by an expression vector.

24. The method according to claim 18, wherein the gp 130 activator is administrated by a recombinant cell expressing the activator.

25. The method according to claim 24, wherein the cells expressing the activator are ES cells.

26. The method according to claim 24, wherein the cells expressing the activator are EB cells.

27. The method according to claim 24, wherein the cells expressing the activator are NS cells.

28. The method according to claim 18, wherein the gp 130 activator is contacted with the cells ex-vivo prior to administration.

29. The method according to claim 18, wherein the gp 130 activator is administrated using an expression vector comprising the coding sequence of the gp 13 activator.

30. The method according to anyone of claims 18 to 29, wherein the gp 130 activator is IL6R/IL6 chimera, a mutein, functional derivative, active fraction, circularly permutated derivative or salt thereof.

31. A method for treating demyelinating diseases, comprising administering to a patient in need thereof an effective amount of one or more gp 130 activators and ES, EB and/or NS cells.

32. A method according to claim 31, wherein the gp 130 activator comprises IL6R/IL6 chimera, a mutein, functional derivative, active fraction, circularly permutated derivative or salt thereof.

33. A method for the preparation of differentiated oligodendrocytes for transplantation into patients in order to repair damage caused by demyelinating diseases comprising contacting one or more gp 130 activator with ES, EB and/or NS cells.

34. A method according to claim 32, comprising ES cells.

35. A method according to claim 32, comprising EB cells.

36. A method according to claim 32, comprising NS cells.

37. The method according to claim 33, wherein the gp 130 activator and the cells are contacted ex-vivo.

38. A method according to claim 33, wherein the gp 130 activator and the cells are contacted in-vivo.

39. A method according to anyone of claims 32 to 38, wherein the gp 130 activator is IL6R/IL6 chimera, a mutein, functional derivative, active fraction, circularly permutated derivative or salt thereof.

40. The use of one or more gp 130 activators, in the manufacture of a medicament for generating oligodendrocytes from ES, EB and/or NS cells, to increase myelination of nerves in injured central neural system (CNS).

41. The use according to claim 40, wherein the cells comprise ES cells.

42. The use according to claim 40, wherein the cells comprise EB cells.

43. The use according to claim 40, wherein the cells comprise NS cells.

44. The use according to claim 40, wherein the gp 130 activator is selected from LIF, CNTF, CT-1, OSM IL-6, IL6R/IL6 chimera and IL-11

45. The use according to claim 44, wherein the gp 130 activator comprises IL6R/IL6 chimera, a mutein, functional derivative, active fraction, circularly permutated derivative or salt thereof.

46. The use according to anyone of claims 40 to 45, wherein injury is caused by demyelination of nerves.

47. The use of one or more gp 130 activators and ES, EB and/or NS cells in the manufacture of a medicament for the treatment of demyelinating diseases.

48. The use according to claim 47, wherein the cells comprise ES cells.

49. The use according to claim 47, wherein the cells comprise EB cells.

50. The use according to claim 47, wherein the cells comprise NS cells.

51. The use according to claim 47, wherein the gp 130 activator is produced by a vector comprising the coding sequence of gp 130 activator.

52. The use according to claim 47, wherein the gp 130 activator is produced by recombinant cells expressing the gp 130 activator.

53. The use according to claim 52, wherein the gp 130 activator is produced by transformed ES, EB and/or NS cells.

54. The use according to anyone of claims 47 to 53, wherein the gp 130 activator is IL6R/IL6 chimera, a mutein, functional derivative, active fraction, circularly permutated derivative or salt thereof.

55. A pharmaceutical composition comprising one or more gp 130 activators and ES, EB and/or NS cells.

56. A pharmaceutical composition comprising an expression vector encoding a gp 130 activator and ES, EB and/or NS cells.

57. A pharmaceutical composition comprising recombinant cells producing one or more gp 130 activators and ES, EB and/or NS cells.

58. A pharmaceutical composition wherein the recombinant cells producing the gp 130 activator are ES, EB and/or NS cells.

59. The pharmaceutical composition according to anyone of claims 55 to 58, comprising ES cells.

60. The pharmaceutical composition according to anyone of claims 55 to 58, comprising EB cells.

61. The pharmaceutical composition according to anyone of claims 55 to 58, comprising NS cells.

62. A pharmaceutical composition according to anyone of claims 55 to 58 for the treatment of a demyelinating disease.

63. A pharmaceutical composition according to anyone of claims 55 to 62, wherein the gp 130 activator is IL6R/IL6 chimera, a mutein, functional derivative, active fraction, circularly permutated derivative or salt thereof.

For the applicant

Henry Einav

Figure 1